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Original Article

Relationship between Intracellular Signaling of the (Pro)renin Receptor and the Pathogenesis of Preeclampsia

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An association between preeclampsia and (pro)renin was recently reported. Intracellular signaling of the (pro)renin receptor [(P)RR] increases the expressions of TGF-β and PAI-1. In this study we sought to clarify the involvement of (pro)renin in the pathogenesis of preeclampsia via the intracellular signaling of (P)RR on preeclampsia placentas. Activated (pro)renin plasma concentrations were compared between pregnant women with (n = 15) and without (n = 28) preeclampsia. The placentas were immunohistochemically evaluated with anti-HIF-1α and anti-(P)RR antibodies. HTR-8/SVneo cells were cultured under hypoxic conditions and treated with human recombinant (pro)renin. The mRNA expressions of HIF-1α, (P)RR, PAI-1, TGF-β, and ET-1 were also examined by real-time RCR. The activated (pro)renin plasma concentration was significantly higher in the third vs. the second trimester in the preeclampsia patients. HIF-1α and (P)RR expressions were significantly increased in the preeclampsia placentas. The mRNA expressions of PAI-1, TGF-β, and ET-1 were significantly increased in the experiments using recombinant (pro)renin vs. hypoxic conditions. (P)RR expression in preeclampsia placentas is increased by persistent hypoxia through the second and third trimesters, and PAI-1, TGF-β, and ET-1 production is increased via (P)RR. Our results suggest that ET-1 production via the intracellular signaling of (P)RR is important in the pathogenesis of preeclampsia.

Key words: preeclampsia, (pro)renin, (pro)renin receptor, endothelin-1, HTR-8/SVneo

Hypertensive disorders of pregnancy (HDPs), including preeclampsia, occur in up to 10% of pregnancies. Preeclampsia is a complication that is involved in maternal and neonatal morbidity and mortality and influences the perinatal prognosis. The pathogenesis of preeclampsia has been described by the two-stage disorder theory proposed by Roberts *et al.* [1]. The onset of preeclampsia is thought to be related to an increase in hypoxia-induced factor-1α (HIF-1α) and increases in antiangiogenic factors such as soluble fms-like tyrosine kinase-1 (sFlt-1) [2,3] and soluble endoglin (sEng) [4,5]. The imbalance between these antiangiogenic and angiogenic factors is important in the onset of preeclampsia, but the pathogenesis of preeclampsia is not yet fully understood.

(Pro)renin is an inactive precursor of renin, and by binding to the (pro)renin receptor [(P)RR], (pro)renin acquires renin enzymatic activity non-proteolytically. The expression of (pro)renin has been confirmed in pregnancy-related tissues such as amniotic fluid, villus, and placenta [6,7]. It was also reported that the blood (pro)renin concentration is higher in the umbilical cord vein than in the umbilical cord artery [8], and that

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(pro)renin is produced in trophoblastic cells during pregnancy [9].

An association between preeclampsia and (pro) renin was reported. Namely, pregnant women with type 1 diabetes and a high concentration of (pro)renin in early pregnancy were shown to be likely to develop preeclampsia [10]. In addition, the (pro)renin placental concentration in women with preeclampsia is significantly higher than that in normotensive pregnant women. However, a previous study reported a lower (pro)renin concentration in women with preeclampsia [11], while another study found no significant difference in the (pro)renin concentration between women with preeclampsia and those with normal pregnancies [12]. The role of the (pro)renin concentration in preeclampsia thus remains unclear.

In this study, we compared the (pro)renin concentration between women with preeclampsia and healthy pregnant women. We immunostained preeclampsia and normal-pregnancy placentas with anti-(P)RR antibody and compared the difference in the placental expression of (P)RR. We also sought to clarify the involvement of (pro)renin in the pathogenesis of preeclampsia via (P)RR-mediated intracellular signaling in the human trophoblast cell line HTR-8/SVneo.

Materials and Methods

Measurement of the activated (pro)renin concentration by ELISA. The subjects were 15 women with preeclampsia and 28 with a normal pregnancy. Maternal plasma samples of the pregnant women who had their delivery at Okayama University Hospital between June 2017 and April 2018 were obtained from routine blood testing at the second and third trimesters. Activated (pro)renin concentrations in maternal plasma samples were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Yanaihara Institute, Shizuoka, Japan) according to the manufacturer's instructions. Samples were assayed in duplicate, and the mean was calculated. Results were compared between the women with preeclampsia and the women with normal pregnancies. Preeclampsia was defined based on the guidelines from the American College of Obstetricians and Gynecologists. The study was approved by the Ethics Committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences and Okayama University Hospital (No. Ken1705-020).

Immunohistochemistry. The human placental tissues of women with preeclampsia and women with normal pregnancies were analyzed by immunohistochemistry. Anti-HIF-1α (Proteintech, Chicago, IL, USA) and anti-(P)RR (Sigma Aldrich, St. Louis, MO, USA) antibodies were used. Briefly, 4-μm-thick paraffin sections from each specimen were mounted on glass slides and dried overnight at 37°C. All sections were deparaffinized in two baths of xylene for 10 min and then rehydrated by sequential incubation with 100%, 95%, 80%, and 60% ethanol for 5 min in each bath. The sections were quenched for endogenous peroxidase activity in 0.3% H_2O_2 for 10 min and processed for antigen retrieval by boiling in citrate buffer (pH 6.0) for 20 min in a 98°C water bath.

The sections were incubated with rabbit anti-HIF-1α (dilution 1 : 100; Proteintech) and rabbit anti-(P)RR (dilution 1 : 200; Sigma Aldrich) polyclonal primary antibodies at room temperature for 60 min. Then, the sections were incubated with secondary antibodies labeled with anti-rabbit horseradish peroxidase polymer (Dako, Glostrup, Denmark) for 30 min. Diaminobenzidine substrate was used as the chromogen for 2 min, and dark brown staining was considered positive. The sections were then counterstained with hematoxylin and observed under a light microscope.

Cell culture. The HTR-8/SVneo cell line (American Type Culture Collection, Manassas, VA, USA) was derived by transfecting the cells that grew out of chorionic villi explants of human first-trimester placenta. HTR-8/SVneo cells were cultured in RPMI-1640 (Sigma Aldrich, St. Louis, MO, USA) containing 5% fetal bovine serum and 1% penicillin/streptomycin/amphotericin B (all from Gibco BRL, Grand Island, NY, USA). The cells were cultured under an atmosphere of humidified 5% CO_2 at 37°C for 24 h (control), or exposed to 5% O_2 hypoxic conditions using a BIONIX hypoxic cell culture kit (Sugiyamgen, Tokyo, Japan) [13, 14], or treated with human recombinant (pro)renin (10 ng/ml; Cayman Chemical, Ann Arbor, MI, USA) [(pro)renin group].

Real-time RT-PCR. For the real-time reverse transcriptase polymerase chain reaction (RT-PCR), total RNA (10 μg) was reverse transcribed in 20 μL of reaction solution according to the protocol of a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The amplification of HIF-1α, (P)RR (encoded by the gene ATP6AP2),

plasminogen activator inhibitor-1 (PAI-1), transforming growth factor-beta (TGF-β), endothelin-1 (ET-1), and β-actin genes was performed. β-actin was used as an internal control. The expression of these genes was detected by using gene-specific TaqMan primers/probes (HIF-1α: Hs00153153_m1; ATP6AP2: Hs00997145_m1; PAI-1: Hs00167155_m1; TGF-β: Hs00998133_m1; ET-1: Hs00174961_m1; β-actin: Hs99999903_m1; Thermo Fisher Scientific, Waltham, MA, USA). The amplification was performed on a STEP ONE PCR system (Applied Biosystems) with initial denaturation at 95°C for 15 s, followed by 50 cycles of annealing at 60°C with a final extension at 60°C for 1 min. The real-time PCR results were obtained by the comparative CT method.

Measurement of ET-1 by ELISA. Maternal plasma samples were obtained from routine blood testing at the third trimester. Serum ET-1 concentrations were measured using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Samples were assayed in duplicate, and the mean was calculated. Results were compared between the pregnant women who developed an HDP and the women with normal pregnancies.

Data analysis. The real-time PCR data are expressed as the mean ± SEM. The data analysis was performed using the Software Package for Social Sciences (IBM, Armonk, NY, USA). The Mann-Whitney *U*-test and χ^2 test were used to examine the between-group differences in concentrations and gene expressions. Differences were considered significant at $p < 0.05$.

Results

Activated (pro)renin concentration in maternal blood. The maternal characteristics of the study participants are summarized in Table 1. In the preeclampsia group (n = 15), the subjects' age and body mass index were significantly higher and weeks of delivery, birth weight, and placental weight were significantly lower ($p < 0.05$). The activated (pro)renin concentration in preeclampsia was significantly lower in the second trimester of pregnancy: 994.8 pg/ml (541.2-2,026.3 pg/ml) in the normal pregnancy vs. 599.4 pg/ml (330.6-896.6 pg/ml) in the preeclampsia group (Fig. 1A). This difference was not significant in the third trimester: 837.6 pg/ml (479.4-2,166.3 pg/ml) in the normal pregnancy vs. 717.0 pg/ml (660.4-1,018.1 pg/ml) in the preeclampsia group (Fig. 1B).

There was no significant difference in the activated (pro)renin concentrations of the normal pregnancy group between trimesters: first trimester vs. second trimester vs. third trimester, 1,225.6 pg/ml (735.5-

Table 1 Maternal characteristics of normal pregnancies and pregnancies with preeclampsia

	Normal pregnancies (n=28)	Preeclampsia (n=15)	*p*-value
Age (years)*	34.0 (23–44)	39.0 (30–43)	$p < 0.05$
Times of pregnancy**			
Primigravida	20 (71.4%)	7 (46.7%)	N.S.
Multigravida	8 (28.6%)	8 (53.3%)	
BMI (kg/m^2)*	20.4 (17.3–32.5)	23.7 (18.2–34.1)	$p < 0.05$
Onset			
Early onset (< 34 week)		7 (46.7%)	
Late onset (≧ 34 week)		8 (53.3%)	
Weeks of delivery (week)*	38.5 (36–41)	35.0 (29–40)	$p < 0.05$
Infant's sex**			
male	12 (42.8%)	6 (40.0%)	N.S.
female	16 (57.2%)	9 (60.0%)	
Birth weight (g)*	3,040 (2,346–3,720)	2,576 (1,040–3,972)	$p < 0.05$
Placental weight (g)*	567 (470–870)	447.5 (258–839)	$p < 0.05$
Methods of delivery**			
Vaginal delivery	16 (57.1%)	6 (40.0%)	N.S.
Caesarean section	12 (42.9%)	9 (60.0%)	

Data are median (range). *Mann-Whitney *U* test, **χ^2 test

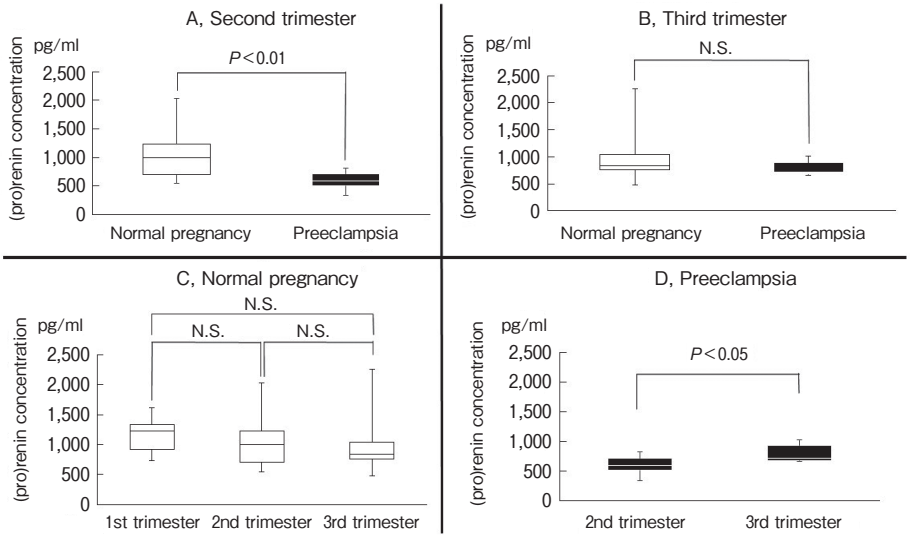

Fig. 1 Activated (Pro) renin concentrations. **A, B**, Comparison of activated (pro)renin concentrations in the second and third trimester between the normal pregnancies and preeclampsia pregnancies; □, Normal pregnancy; ■, Preeclampsia pregnancy; **C, D**, Comparison of activated (pro)renin concentration between the normal pregnancies and preeclampsia pregnancies in each trimester; □, Normal pregnancy; ■, Preeclampsia pregnancy. $^{*}p < 0.05$.

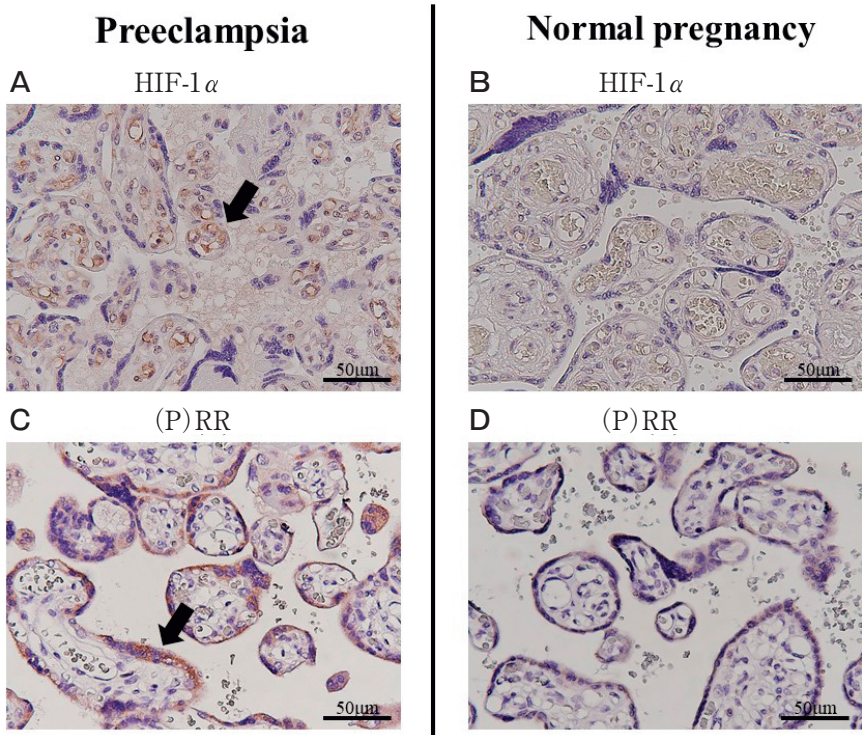

Fig. 2 Immunohistochemical staining of HIF-1*α* and (P)RR at 400× magnification. Positive staining is shown in brown (*black arrow*). **A**, Immunohistochemical staining of HIF-1*α* in preeclampsia placentas; **B**, Immunohistochemical staining of HIF-1*α* in normal pregnancy placentas; **C**, Immunohistochemical staining of (P)RR in preeclampsia placentas; **D**, Immunohistochemical staining of (P)RR in normal pregnancy placentas.

1,613.0 pg/ml) vs. 994.8 pg/ml (541.2-2,026.3 pg/ml) vs. 837.6 pg/ml (479.4-2,166.3 pg/ml) (Fig. 1C). However, the activated (pro)renin concentrations were significantly higher in the third trimester than in the second trimester in the preeclampsia group: second trimester vs. third trimester, 599.4 pg/ml (330.6-896.6 pg/ml) vs.

717.0 pg/ml (660.4-1,018.1 pg/ml) (Fig. 1D).

Immunohistochemical staining of HIF-1α and (P)RR in human placental tissues. Preeclampsia placentas are hypoxic, and we thus performed immunohistochemical staining for HIF-1α in human placental tissues. To confirm that the expression of (P)RR is increased under hypoxic conditions, we also performed the immunohistochemical staining of (P)RR.

The expression of HIF-1α was increased in the preeclampsia placentas (Fig. 2A) compared to that in the women with normal pregnancies (Fig. 2B). The expression of (P)RR was also increased in the preeclampsia placentas (Fig. 2C) compared to that in the women with normal pregnancies (Fig. 2D). The proportion of a moderate-to-strong expression of (P)RR was significantly higher in the preeclampsia placentas: women with normal pregnancies vs. women with preeclampsia, 2/12 (16.7%) vs. 13/17 (76.4%).

The mRNA expressions of HIF-1α, (P)RR, PAI-1, TGF-β, and ET-1 in HTR-8/SVneo cells under hypoxic conditions. We performed experiments using HTR-8/SVneo cells to investigate the role of intracellular signaling via (P)RR. The mRNA expressions of all targets were significantly higher in the hypoxic conditions than in the controls cultured for 24 h (Fig. 3). The control value in this experiment was set to 1, and the value of hypoxic conditions was calculated as the ratio to the control value.

The mRNA expressions of PAI-1, TGF-β, and ET-1 in HTR-8/SVneo cells treated with human recombinant (pro)renin under hypoxic conditions. The mRNA expressions of all targets were significantly higher in the (pro)renin group than in the hypoxic conditions cultured for 24 h (Fig. 4A-C). The hypoxic condition value in this experiment was set to 1, and the value of the (pro)renin group was calculated as the ratio to the hypoxic condition value.

ET-1 concentration in maternal blood. To determine whether ET-1 is also elevated *in vivo*, we measured the ET-1 concentration in maternal blood. In the third trimester of pregnancy, the ET-1 concentration in the preeclampsia group at 1.30 pg/ml (0.16-2.78 pg/ml) was significantly higher than that in the women with normal pregnancies at 0.66 pg/ml (0.23-1.48 pg/ml) (Fig. 5).

Discussion

In preeclampsia, the vascular resistance of the

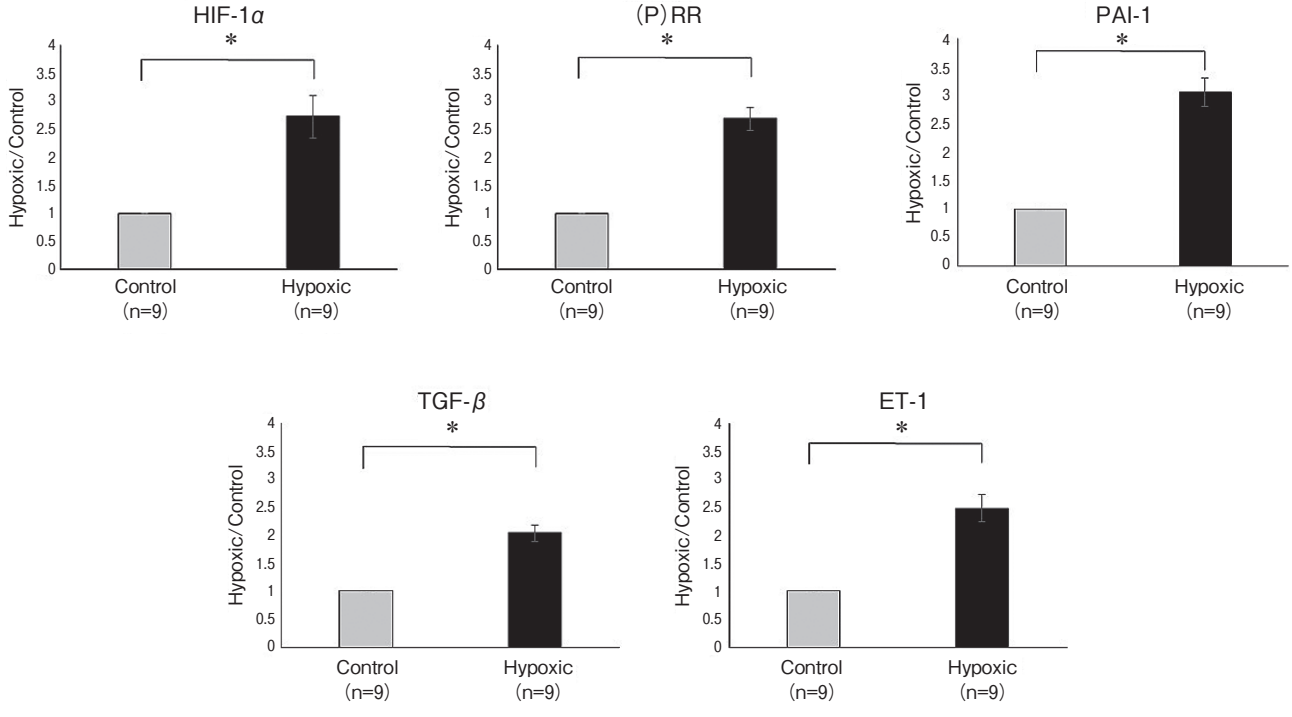

Fig. 3 The mRNA expressions of HIF-1α, (P)RR, PAI-1, TGF-β and ET-1 under hypoxic conditions. *Gray bars*: Control. *Black bars*: Hypoxic conditions. Values are mean ± SEM. $^*p < 0.05$.

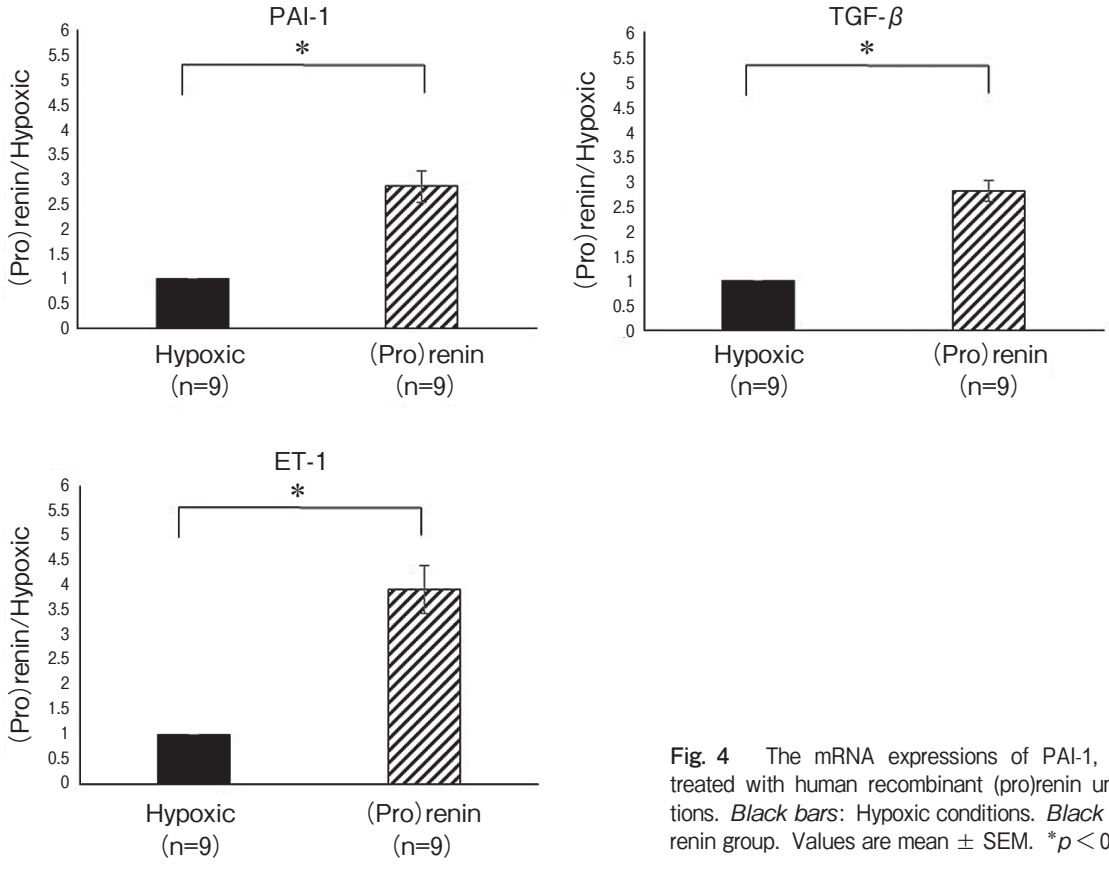

Fig. 4 The mRNA expressions of PAI-1, TGF-β, and ET-1 treated with human recombinant (pro)renin under hypoxic conditions. *Black bars*: Hypoxic conditions. *Black shaded bars*: (pro) renin group. Values are mean ± SEM. $^{*}p < 0.05$.

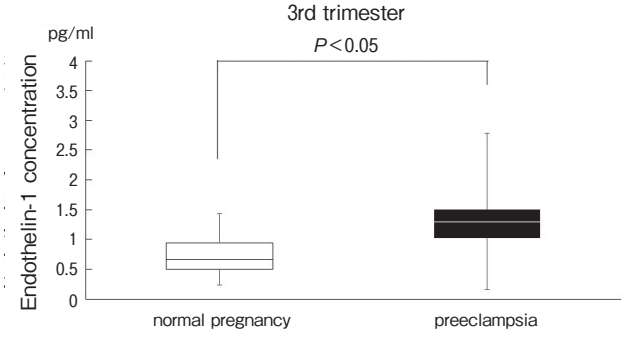

Fig. 5 Comparison of ET-1 concentration in the third trimester between the normal and preeclampsia pregnancies. □, Normal pregnancy; ■, Preeclampsia pregnancy. $^{*}p < 0.05$.

maternal uterine spiral artery does not decrease due to inadequate remodeling in the early stage of pregnancy, and this hypoxic condition persists in preeclampsia placentas [15]. As a result of persistent hypoxia due to placental blood flow disturbance, the preeclampsia placenta becomes hypoplastic [16, 17]. In the present study, the activated (pro)renin concentration in the preeclampsia patients was significantly lower than in the women with normal pregnancies in the second trimester. However, we observed the opposite results in the third trimester. Compared to the production in women with normal pregnancies, the production of activated (pro)renin in the preeclampsia placenta is reduced because of placental hypoplasia. However, as the hypoxia condition persists in preeclampsia, the expressions of HIF-1α and (P)RR in the placenta are increased, and thus the activated (pro)renin concentration in maternal blood is increased in the third trimester. It has been reported that the expressions of HIF-1α [18] and (P)RR [19] are increased in the preeclampsia placenta. In our present study, immunohistochemistry was performed on the human placental tissue using anti-HIF-1α and anti-(P)RR antibodies. Both expressions were increased in the women with preeclampsia compared to the women with normal pregnancies.

We also examined the involvement of the pathogenesis of preeclampsia via (P)RR-mediated intracellular

signaling in the human trophoblast HTR-8/SVneo cell line, and we observed that the mRNA expressions of HIF-1α, (P)RR, PAI-1, TGF-β, and ET-1 were significantly increased in the HTR-8/SVneo cells under hypoxic conditions. Moreover, the mRNA expressions of PAI-1, TGF-β, and ET-1 were significantly increased in the HTR-8/SVneo cells treated with human recombinant (pro)renin under hypoxic conditions. It has been reported that the expression of (P)RR is increased in chorionic cells under hypoxic conditions [19]. We obtained similar results in the hypoxic HTR-8/SVneo cell cultures in this study.

(P)RR-mediated intracellular signaling has been shown to activate mitogen-activated protein kinase and extracellular signal-regulated kinase 1/2 [20-23], and it was observed that the expressions of PAI-1 and TGF-β are increased through their intracellular signaling [24,25]. TGF-β is a cytokine that causes the production of ET-1; in the present study, the ET-1 production in HTR-8/SVneo cells increased. ET-1 is a potent vasoconstrictor, and a relationship between ET-1 and preeclampsia has been described in recent years [26-29]. The ET-1 maternal plasma level is increased in preeclampsia [26]. ET-1 has been reported to correlate with antiangiogenic factors such as sFlt-1, and it has been considered to play an important role in the pathogenesis of preeclampsia [27]. By the binding of (pro) renin to (P)RR in chorionic cells, the production of PAI-1 and TGF-β increases through intracellular signaling. As a result, the production of ET-1 increases, and this increase is thought to contribute to the onset of hypertension in preeclampsia.

The activated (pro)renin concentration during pregnancy depends on the placenta, and its concentration in the second trimester in preeclampsia is low due to placental hypoplasia. However, the expression of (P) RR is increased by the persistence of hypoxia due to uteroplacental circulation failure, and the activated (pro)renin concentration is increased in the third trimester. An increased expression of (P)RR in the placenta of preeclampsia activates intracellular signaling via (P)RR, resulting in increased productions of PAI and TGF-β. The production of ET-1 consequently increases, and it has been speculated that this increase contributes to the development of hypertension. Our present analyses revealed a relationship between the (pro)renin concentration during pregnancy in preeclampsia and the expression of (P)RR in the placenta. This report is also the first to clarify the importance of the production of ET-1 via the intracellular signaling of placental (P)RR in the pathogenesis of preeclampsia.

As a study limitation, we did not investigate the relation between (pro)renin and antiangiogenic factors and angiogenic factors, and it is unclear how much ET-1 is involved in hypertension in preeclampsia. Further studies *in vivo* are required to determine whether suppressing the production of ET-1 inhibits hypertension.

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